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STUDY OF TOXIC AND ANTIGENIC STRUCTURES OF BOTULINUM NEUROTOXINS

Annual Report

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The neurotoxin (NT) from type B (strain 657) was partially sequenced (44 residues from light chain and 26 residues from heavy chain). Significant sequence homology between this type B botulinum and tetanus NTs are now evident. A second procedure was developed to enzymatically cleave the heavy chain of type A NT into two halves; one of these fragments was partially sequenced. Secondary structures of types A and E NT in terms of α helix and β sheet contents were determined. Changes in conformation (hydrophobicity) were detected when single chain type E NT was nicked to the dichain form. Roles of i) Lys residues in types A and B NT, and ii) Tyr residues in types A, B and E NT were studied based on chemical modification and quantitative assay of amino acid modification, toxicity and change in serological activity. Type B NT completely detoxified following modification of Tyr residues was a good immunogen (second generation toxoid). Keywords: Amino acid sequence. (AU)

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SUMMARY

The type B neurotoxin (NT) isolated from Clostridium botulinum (strain 657) behaved as a mixture of single (unnicked) and dichain (nicked) protein both of M_r ~150K. When reduced with mercaptoethanol the two chains from the dichain NT were separated and migrated in SDS-PAGE as polypeptides of M_r 100 and 50K similar to the H and L chains of other serotypes of botulinum NT. The N-terminal sequences of the two chains were determined. A procedure, different from that reported earlier, was developed to produce and purify the two halves of the H chain of type A NT. Amino acid composition and partial amino acid sequence of the C-terminal half was determined. Conformations of dichain type A (nicked by endogenous protease), single chain type E and dichain type E (nicked by trypsin) were compared at different pHs using circular dichroism and fluorescence spectroscopy. Roles of Lys residues in types A and B NTs and Tyr residues in types A, B and E NTs (with respect to their structure-function relationship) were studied based on chemical modification and quantitative assay of amino acid modification, toxicity and change in serological activity. Type B NT completely detoxified following modification of Tyr residues proved to be a good immunogen (second generation toxoid) in rabbits. The antiserum had good NT neutralizing titer.

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHHS Publication No. (NIH) 86-23, Revised 1985).

BODY OF REPORT

Clostridium botulinum has been a subject of awe and alarm because the ubiquitous spore forming bacteria produce the protein, botulinum neurotoxin, generally regarded as the most poisonous poison (2×10^{-8} mg is lethal for a mouse) made by nature, in antigenically distinguishable forms (types A, B, C, D, E, F, and G). The neurotoxin (NT) causes the dreaded neuroparalytic disease botulism (1).

Definitive diagnosis and the only effective therapy for botulism depends, at present, on a chemically modified form(s) of the neurotoxin i.e. toxoid (immunogen) and the antiserum raised against the immunogen. Therapy for botulism is supportive care following neutralization of the circulating NT with type specific antiserum. Protection against botulism through prophylactic immunization is confined only to the laboratory personnel who work with C. botulinum or the NT. A better immunogen, free of side effects, would provide effective prophylactic protection for a population mass (civilians and military) facing potential exposure to the deadly NT (1,2). Mass immunization with tetanus toxoid has essentially wiped out tetanus as a public health problem. The structures of tetanus and botulinum neurotoxins are very similar yet active immunity against tetanus lasts up to about 10 years while booster shots of botulinum toxoid are administered every 2-3 years to maintain good immunity. Crucial for diagnosis and therapy are the type specific antisera and therefore the type specific immunogens. Until about 1960 only types A-E were known. Gradual recognition of types F and G indicates that other unrecognized types are probably present in nature to which we are vulnerable. An unusual type B NT (from strain 657, isolated from human infant botulism) was identified; it is ~5000 x more toxic to infant than adult mice, and it requires ~1000 fold more standard type B antiserum for neutralization than the well known type B NT (3). These facts show our poor understanding of the immunogenic nature of the botulinum NT.

This research activity, part of a long term, ongoing project, was on the i) elucidation of the structure of botulinum NTs, ii) determination of structure-antigenicity relationships, iii) preparation of immunogens (second generation immunogens, see p. 6 for explanation) better than the traditional toxoids.

During this contract period our work covered, i) purification and characterization of the unusual type B NT from strain 657, ii) preparation of second generation toxoids, iii) fragmentation of NT, isolation and characterization of the fragments, iv) determination of amino acid sequence and v) determination of antigenic structures (selective modification of amino acid residues. We initiated examination of conformation based on spectrophotometric and fluorometric methods.

These separate and independent studies are presented in five separate sections each with its experimental method, results, discussions and conclusions.

1. CHARACTERIZATION OF THE NEUROTOXIN FROM TYPE B STRAIN 657:

The strain 657, isolated from a case of human infant botulinum, produced type B toxin that appeared unusual in its serological properties; at least two species of toxic proteins were thought to be involved of which only one corresponds to the type B specificity (Hatheway et al. J. Clin. Microbiol. 14, 607 [1981]). It was also reported that the 657 toxin is a mixture of type B (~95%) and type A toxins and the type B toxin from strain 657 represents the prototype of a new serotype named subtype Ba (Gimenez Zbl. Bakt. Hyg. A. 257, 68 [1984]). This conclusion was based on cross-neutralization tests done with anti-serum prepared against neurotoxin serotypes A, B and B-657.

We purified the 657 toxin following a method of purification of type A rather than that of type B (strain Okra) neurotoxin (NT). Following its purification, the 657 toxin was identified serologically by testing against antitype A and antitype B (strain Okra) NT sera. Also the two chains of the 657 toxin were separated and analyzed for amino acid sequence.

The toxin behaved as a mixture of single (unnicked) and dichain (nicked) protein, both of ~150Ka. When the protein was reduced with mercaptoethanol the two chains migrated in SDS-PAGE as separate polypeptides of M_r ~100K and 50K that appeared similar to the heavy and light chains of other serotypes of botulinum NT. The N-terminal amino acid sequences of the two chains were determined. They were as follows; light chain: Pro.Val.Thr.Ile.Asn.Asn.Phe.Asn.Tyr.Asn.Asp.Pro.Ile.Asp.Asn.Asn.Asn.Ile.Ile. Met.Met.Glu.Pro.Pro.Phe.Ala.Arg.Gly.Met.Gly.Arg.Tyr.Tyr.Lys.Ala.Phe.Lys.Ile. Thr.Asp.Arg.Ile.Trp.Ile.-; and heavy chain: Ala.Pro.Gly.Ile.X.Ile.Asp.Val. Asp.Asn.Glu.Asp.Leu.Phe.Phe.Ile.Ala.Asp.Lys.Asn.Ser.Phe.Arg.Asp.Asp.Leu.- These two sequences matched exactly with those of the light and heavy chains of type B NT (strain Okra) of which only 16 and 18 residues were known (Sathyamoorthy and DasGupta J. Biol. Chem. 260, 10461, 1985). The above sequences were different from those of type A NT. Immunoprecipitation reactions of type B NT isolated from strains 657 and Okra were indistinguishable against polyclonal antitype B NT serum. These two preparations did not produce precipitin reactions with polyclonal antitype A NT serum. The tetanus NT (from Clostridium tetani) resembles botulinum NT in relative molecular mass, dichain structure and probably in the mode of action; but the two NTs differ in their sites of action and their serologic specificity. Comparison of amino acid sequences of type B botulinum NT, reported here, and tetanus NT (Eisel et al. EMBO J. 5, 2495, 1986) shows that their two heavy chains are less homologous (13 out of 26 residues matched in position) than the two light chains (31 out of 44 residues matched). Three stretches residues #3-7, 18-24 and 32-44 on the light chains of the two NTs are identical.

2. ENZYMATIC CLEAVAGE OF THE HEAVY CHAIN AND PARTIAL CHARACTERIZATION OF THE TWO FRAGMENTS:

The two halves, i.e. the N- and C-terminal halves, of the heavy chain of the NT is thought to have different functions in the mechanism of action of the NT (by analogy with diphtheria and tetanus toxin). Well characterized and highly purified preparations of the two halves of the heavy chain are needed

for such studies. Two different approaches were taken to cut the heavy chain with trypsin and isolate the fragments. In one method, developed earlier, the intact type A NT (150 KDa) was digested with trypsin to cut the heavy chain. The cleavage products were: i) 94 KDa fragment made of the L chain linked to the N-terminal half of the heavy chain (49 KDa) by a disulfide bond(s), and ii) the C-terminal 44 KDa fragment. The second method developed was as follows: The heavy chain of type A NT was first separated from the light chain, purified and then digested with trypsin. One product of cleavage, the 44 KDa fragment (C-fragment) was chromatographically purified and then analyzed for amino acid composition and sequence. The sequence, given below, matches with the sequence of the C-fragment (only 12 residues were sequenced) obtained by method one (i.e. digestion of intact NT) except at positions 1 and 3.

```
1       5         10        15        20
S-I-R-N-L-G-I-L-N-L-R-Y-E-V-N-D-L-I-D-L-R-Y-Y-H-
```

This work also demonstrated that in type A i) the cysteine residues located on the N-terminal half of the heavy chain forms the -S-S- link(s) with the light chain. ii) The C-terminal half (44 KDa fragment) is not linked via -S-S- to the L-chain or to the N-terminal half (49 KDa fragment) of the heavy chain.

Digestion of intact type E NT with trypsin also cleaved the heavy chain at about the mid-point. One cleavage product was the light chain linked to the N-terminal half of the heavy chain via disulfide bond(s), hence its total size ~100 KDa. This fragment of the heavy chain was separated from the light chain, chromatographically purified and partially sequenced. Two independent sequence runs gave a sequence (see below) which is identical to the known N-terminal sequence of the intact heavy chain. This confirms that the isolated and purified fragment is the N-terminal half of the heavy chain.

```
1       5         10
K-S-I-C-I-E-I-N-N-G-E-L-F-
```

Tryptic fragmentation at about the mid-point of the heavy chain of type B NT has been accomplished at the analytical level.

3. STRUCTURE-FUNCTION RELATIONSHIP:

The role of amino acid residues as structural components of biologically active sites of the NT were examined by specific chemical modification of lysine and tyrosine residues.

Lysine: Type A and B NTs were modified by reductive methylation using $NaBH_4$ and formaldehyde at different molar ratios of the protein and the reagent and then they were analyzed for change in toxicity, serological reactivity and the number of lysine residues modified. Amino acid analysis of the modified proteins (HCl hydrolysed) confirmed selective modification of lysine. The derivative N,N-dimethyl lysine was more abundant than monomethyl lysine; trimethyl lysine was not detected. Distribution of modified lysine residues among the heavy and light chains of the dichain type A NT was approximately proportional to the lysine contents of the two subunits of the NT. Toxicity (mouse lethality) and serological reactivity (polyclonal

antibody) of type A NT were not (or insignificantly) damaged following methylation of up to 72 lysine residues. Modification of 3 additional residues caused precipitious loss in toxicity. The type B NT appeared more susceptible to the effect of lysine modification than type A NT. When 31, 55, 77 or 88 lysine residues of type B NT (out of a total of 118 residues) were modified, mouse death time (31 min for control) was found to be 47, 79, 143 or 186 min, respectively. Difference between type A and B NTs with respect to the effect of modification on toxicity suggests that role of lysine residues in structure-function relationship in these two antigenically distinct proteins may not be identical.

Tyrosine. Type A, B and E NTs were modified at pH 7.9 with tetranitromethane, a reagent highly specific for tyrosine residues. Modification of only tyrosine residues to nitrotyrosine was evident from amino acid analysis of the acid hydrolysates of the modified proteins. The type B and E NTs could be completely detoxified without causing significant damage to their serological reactivities. Under similar modification reaction conditions, the type A NT was incompletely detoxified with some alteration in tis serological reactivity. Following relationship between molar excess of reagent over NT, number of nitrotyrosine residues formed and loss in toxicity emerged:

TNM molar excess	Type A NT		Type B NT		Type E NT	
	Nitro-Tyr.*	% loss toxicity	Nitro-Tyr.	% loss toxicity	Nitro-Tyr.	% loss toxicity
500	19.7	<100	ND	100	24.5	100
125	--	99	--	--	--	--
100	ND	95	<1.0	>98	7.6	>99
80	--	--	1.2 (?)	91	--	--
50	ND	85	--	--	--	>90
40	--	--	trace	70	--	--
0	0	0	0	0	0	0

* - number of nitro-tyrosine residues detected
ND = is not detectable, Number of Tyr residues in types A, B, and E NTs are 71, 81, 70, respectively.

4. SECOND GENERATION TOXOID:

The completely detoxified type B and E NTs, used as toxoid, elicited antibodies in rabbits. The antiserum precipitated and neutralized the homologous NT. Two separate batches of each of these toxoids were prepared for immunizing rabbits in separate groups. The two toxoids, type B and E, were prepared with >99% pure NTs as tested by SDS-PAGE whereas the traditional toxoids produced with formaldehyde are very crude preparations of the NT (~90% impure). Chemical modification induced by tetranitromethane is more specific than the products that form during ~7 days of reaction between a

protein and formaldehyde. The toxoids produced with tetranitromethane may be considered as a second-generation toxoid, compared with the first-generation toxoid (crude preparation of NT detoxified with formaldehyde).

5. TERTIARY STRUCTURE OF NT:

The conformations of dichain type A (nicked by endogenous protease), single chain type E, and dichain type E NT (nicked by trypsin) were compared at different pHs using circular dichroism (CD) and fluroescence spectroscopy. The high degree of ordered secondary structure (α helix 28%, β sheet 42%, total 70%) found in type A NT at pH 6.0 was similar to that found at pH 9.0 (α 22%, β 47%, total 69%). The secondary structure of the single chain type E NT at pH 6.0 (α 18%, β 37%, total 55%) differed somewhat from these values at pH 9.0 (α 22%, β 43%, total 65%). The dichain type E NT at pH 6.0 assumed a secondary structure (α 20%, β 47%, total 67%) that seemed more similar to that of dichain type A than the single chain type E NT. Examination with the fluorogenic probe toluidine napthalene sulfonate revealed that the hydrophobicity of the type A and E NTs were higher at pH 9.0 than at pH 6.0. Also, the hydrophobicity of the dichain type E NT was higher than its precursor the single chain protein and appeared similar to that of the dichain type A NT. The CD and fluorescence studies indicate that conversion of the single chain type E NT to the dichain form (i.e. nicking by trypsin) induced changes in conformation.

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